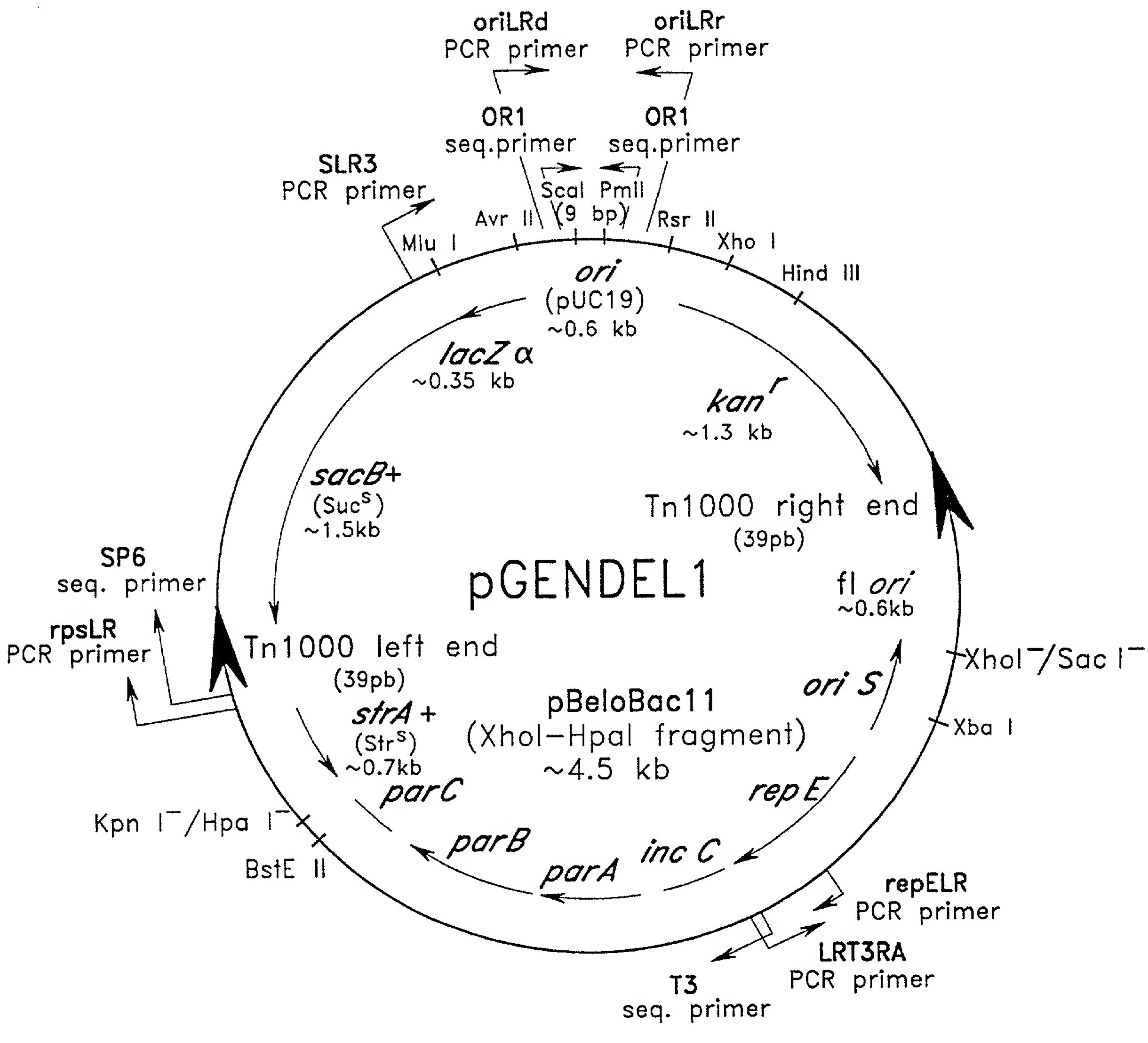
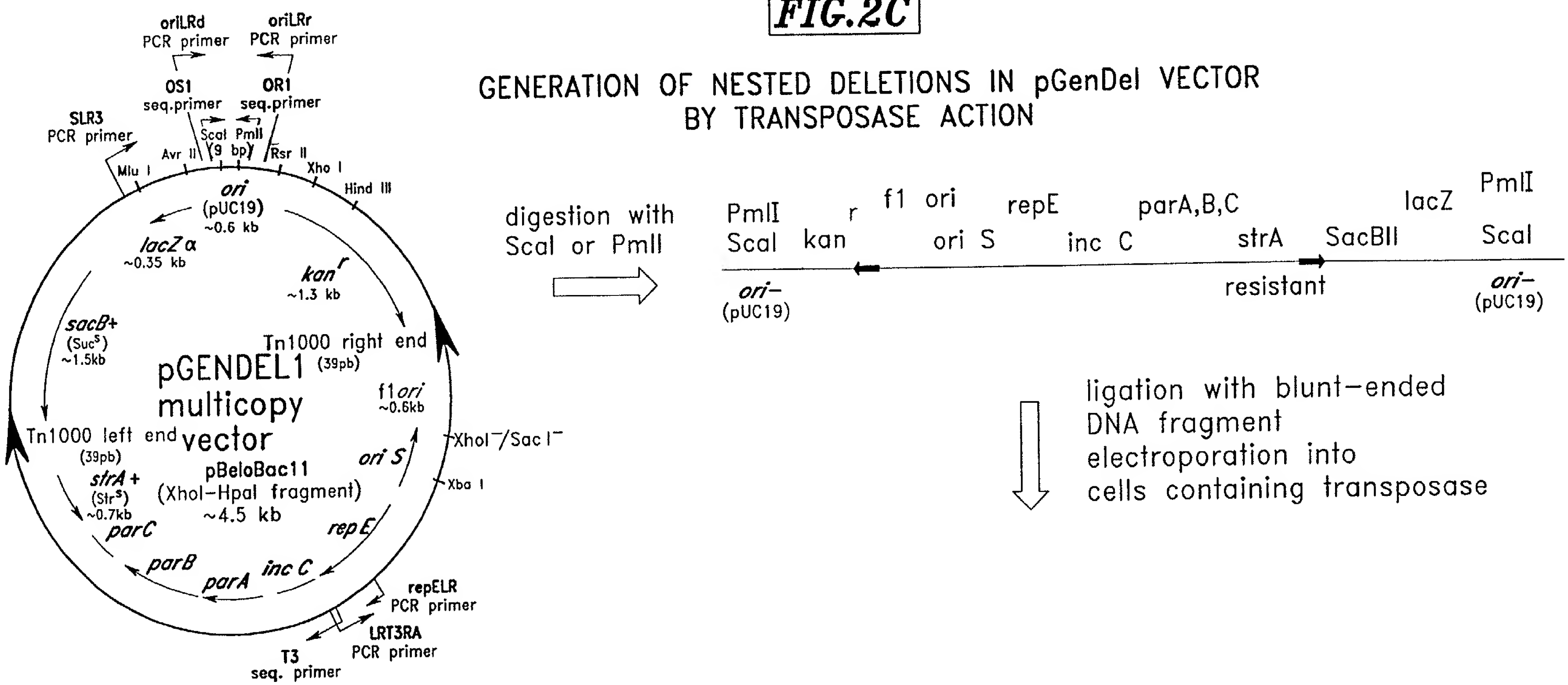

oriLRd
PCR primer
oriLRr
PCR primer
OR1
seq.primer
OR1
seq.primer
SLR3
PCR primer
ScaI
PmlI
(9 bp)
Mlu I
Avr II
Rsr II
Xho I
Hind III
ori
(pUC19)
~0.6 kb
lacZ α
~0.35 kb
kan^r
~1.3 kb
sacB+
(Suc^S)
~1.5kb
Tn1000 right end
(39pb)
pGENDEL1
fl ori
~0.6kb
SP6
seq. primer
rpsLR
PCR primer
Tn1000 left end
(39pb)
XhoI⁻/Sac I⁻
ori S
strA +
(Str^S)
~0.7kb
pBeloBac11
(XhoI–HpaI fragment)
~4.5 kb
Xba I
parC
rep E
Kpn I⁻/Hpa I⁻
parB
inc C
parA
BstE II
repELR
PCR primer
LRT3RA
PCR primer
T3
seq. primer

FIG. 1

FIG.2

FIG.2A
FIG.2B
FIG.2C

GENERATION OF NESTED DELETIONS IN pGenDel VECTOR
BY TRANSPOSASE ACTION



kanamycine resistant, streptomycine sensitive if introduced into streptomycine resistant host cells, sucrose sensitive deeply on IPTG/Xgal plates

FIG.2A

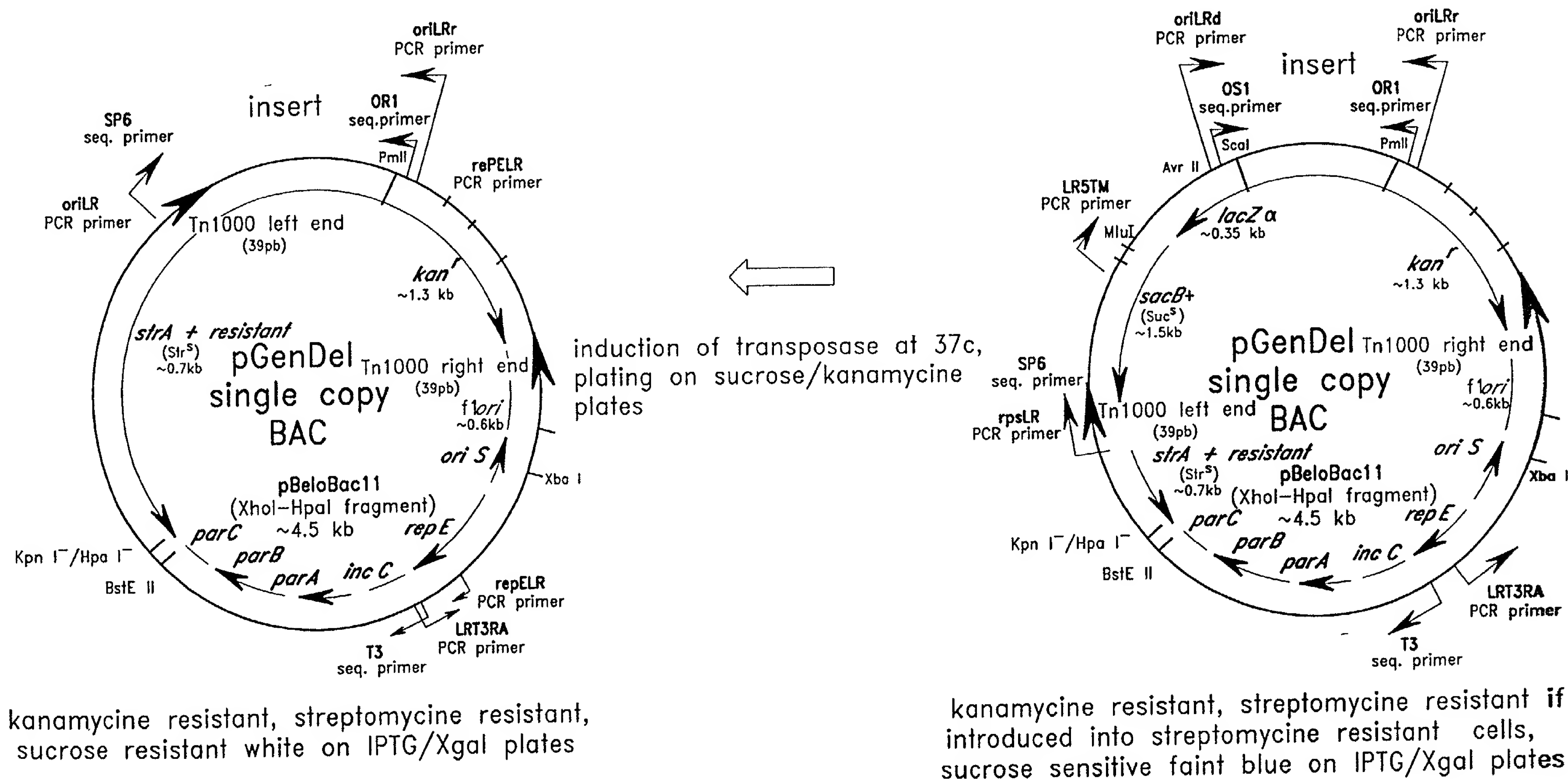
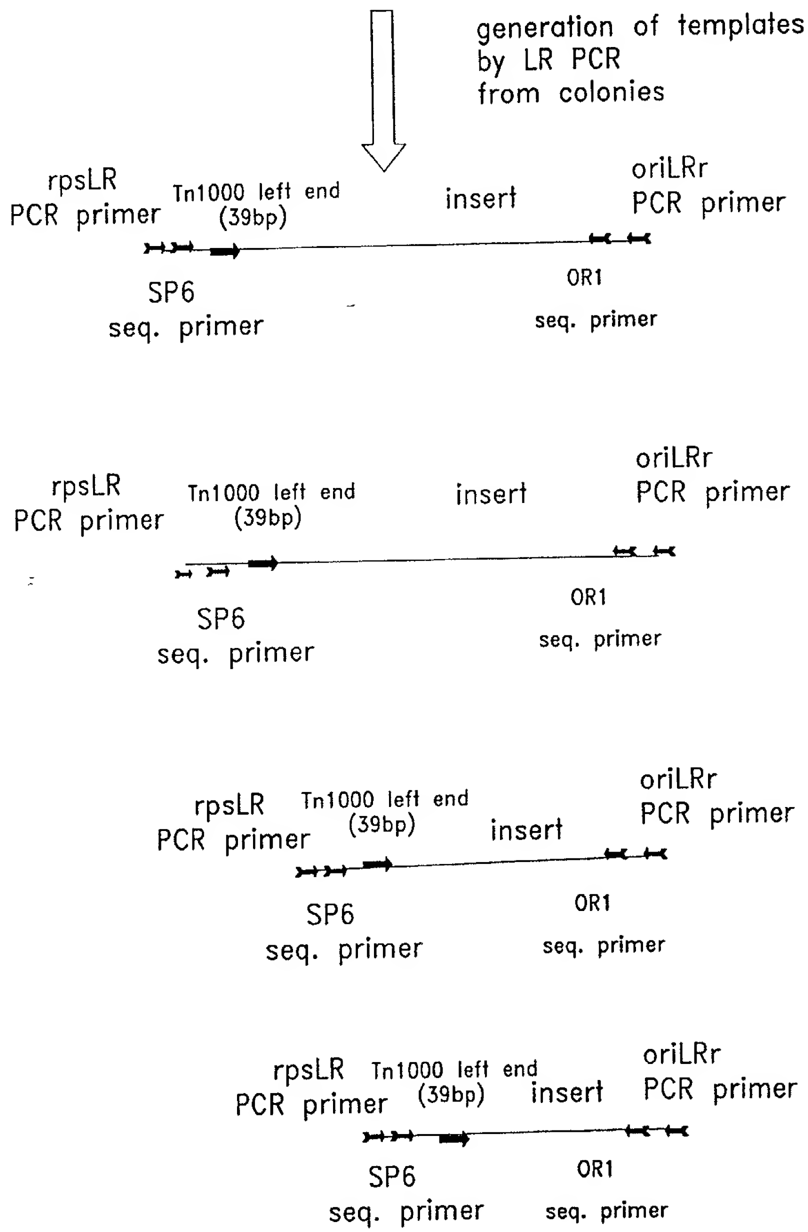

oriLRd
PCR primer
insert
oriLRr
PCR primer
OS1
seq.primer
OR1
seq.primer
ScaI
PmlI
Avr II
LR5TM
PCR primer
MluI
lacZ α
~0.35 kb
kan^r
~1.3 kb
sacB+
(Suc^S)
~1.5kb
pGenDel
single copy
BAC
Tn1000 right end
(39pb)
f1ori
~0.6kb
SP6
seq. primer
rpsLR
PCR primer
Tn1000 left end
(39pb)
strA + resistant
(Str^S)
~0.7kb
pBeloBac11
(XhoI–HpaI fragment)
~4.5 kb
ori S
Xba I
repE
parC
parB
parA
inc C
Kpn I^−/Hpa I^−
BstE II
LRT3RA
PCR primer
T3
seq. primer
kanamycine resistant, streptomycine resistant if introduced into streptomycine resistant cells, sucrose sensitive faint blue on IPTG/Xgal plates
induction of transposase at 37c, plating on sucrose/kanamycine plates
oriLRr
PCR primer
insert
OR1
seq.primer
PmlI
SP6
seq. primer
rePELR
PCR primer
oriLR
PCR primer
Tn1000 left end
(39pb)
kan^r
~1.3 kb
strA + resistant
(Str^S)
~0.7kb
pGenDel
single copy
BAC
Tn1000 right end
(39pb)
f1ori
~0.6kb
ori S
Xba I
pBeloBac11
(XhoI–HpaI fragment)
~4.5 kb
parC
repE
parB
parA
inc C
Kpn I^−/Hpa I^−
BstE II
repELR
PCR primer
LRT3RA
PCR primer
T3
seq. primer
kanamycine resistant, streptomycine resistant, sucrose resistant white on IPTG/Xgal plates

FIG.2B



1.Forced cloning of blunt ended fragments into pGenDel by contra-selection on streptomycine plus kanaycine

2. Selection of intra transposed clones by plating on sucrose/kanamycine/Xgal media

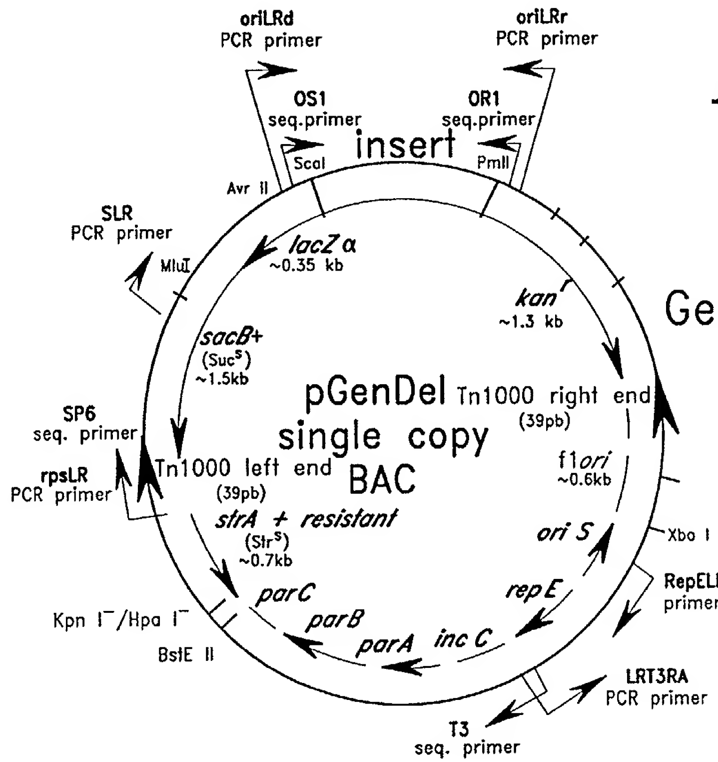
3. Generation of templates by PCR from colonies.

4. Minimal tiling path determination by sizing

FIG.2C

FIG. 3

FIG. 3A
FIG. 3B
FIG. 3C



Generation of nested deletions with exoIII

kanamycine resistant, streptomycine resistant if introduced into streptomycine resistant cells, sucrose sensitive, faint blue on IPTG/Xgal plates

generation of linear substrates by LR PCR with SLR3 and LRT3RA primers from cells

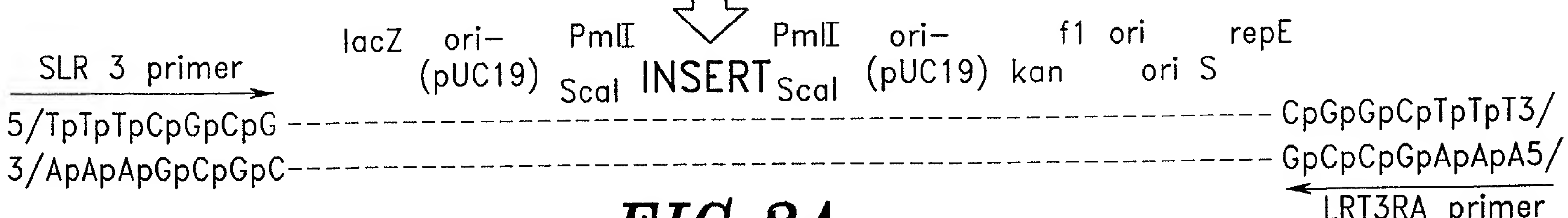


FIG. 3A

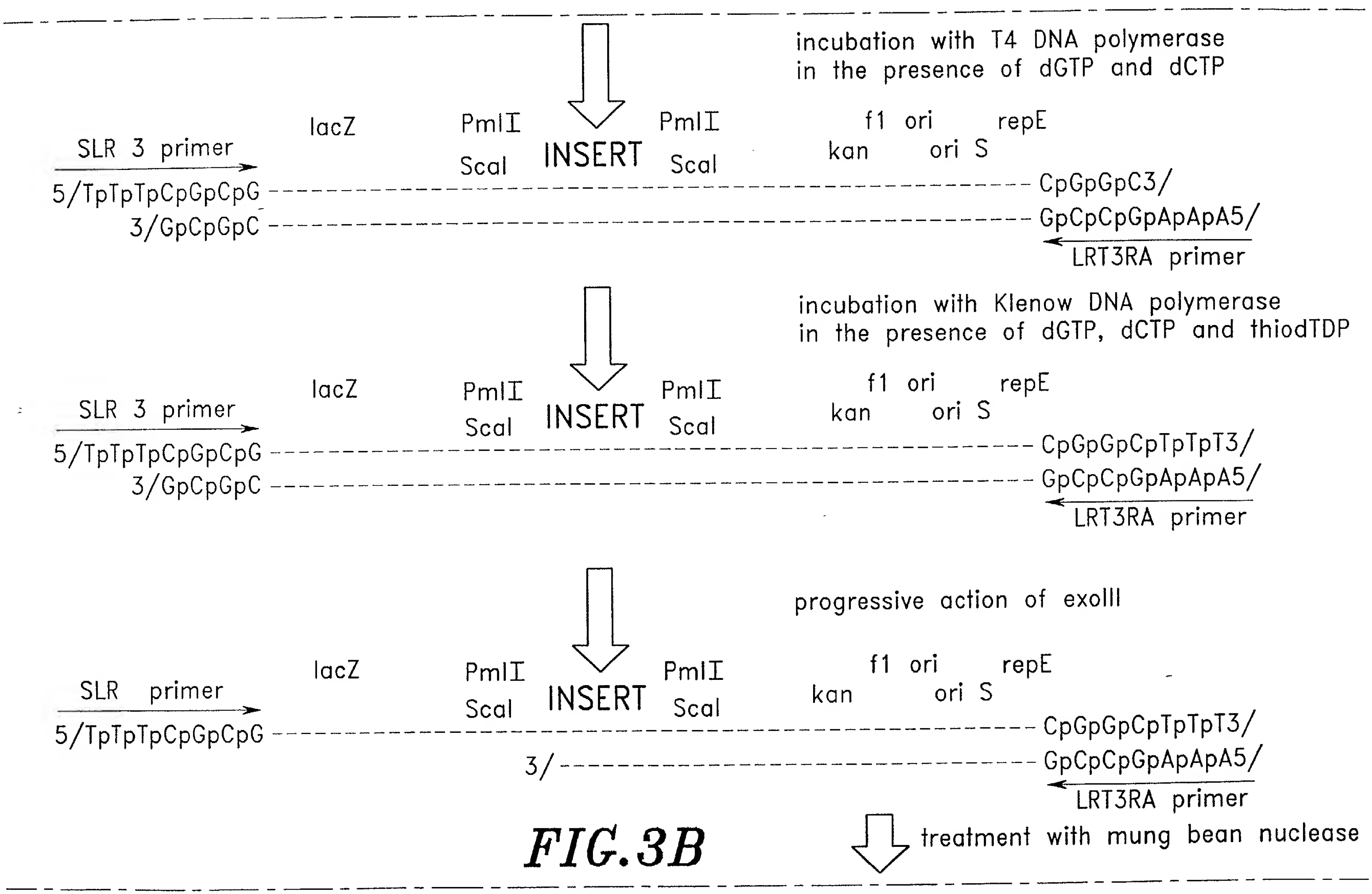
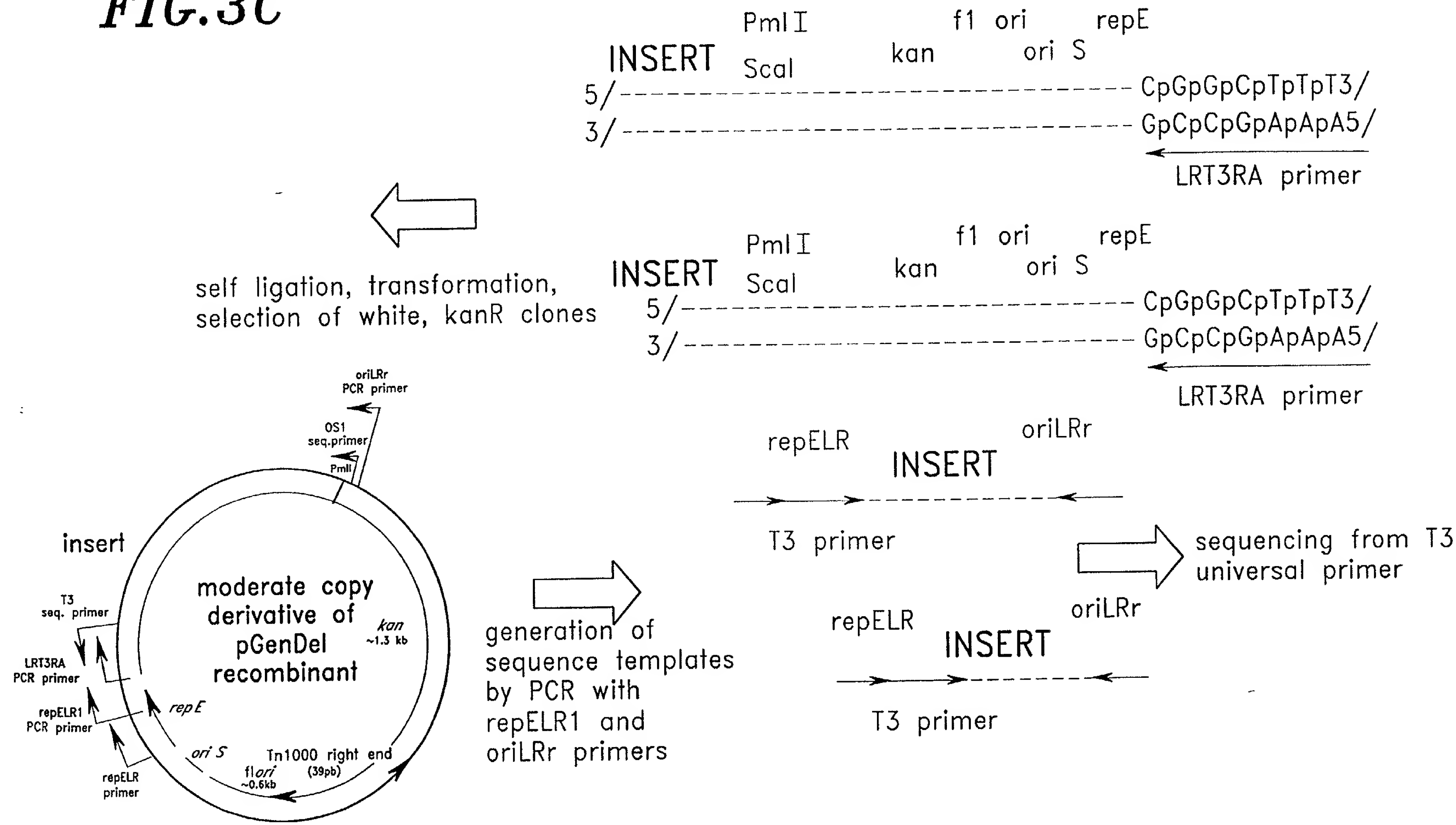

incubation with T4 DNA polymerase
in the presence of dGTP and dCTP
lacZ
PmlI
ScaI
INSERT
PmlI
ScaI
f1 ori
repE
kan
ori S
SLR 3 primer
5/TpTpTpCpGpCpG
3/GpCpGpC
CpGpGpC3/
GpCpCpGpApApA5/
LRT3RA primer
incubation with Klenow DNA polymerase
in the presence of dGTP, dCTP and thiodTDP
lacZ
PmlI
ScaI
INSERT
PmlI
ScaI
f1 ori
repE
kan
ori S
SLR 3 primer
5/TpTpTpCpGpCpG
3/GpCpGpC
CpGpGpCpTpTpT3/
GpCpCpGpApApA5/
LRT3RA primer
progressive action of exoIII
lacZ
PmlI
ScaI
INSERT
PmlI
ScaI
f1 ori
repE
kan
ori S
SLR primer
5/TpTpTpCpGpCpG
3/
CpGpGpCpTpTpT3/
GpCpCpGpApApA5/
LRT3RA primer
treatment with mung bean nuclease

FIG.3B

FIG.3C



kanamycine resistant, streptomycine resistant if introduced into streptomycine resistant cells, sucrose sensitive, white on IPTG/Xgal plates

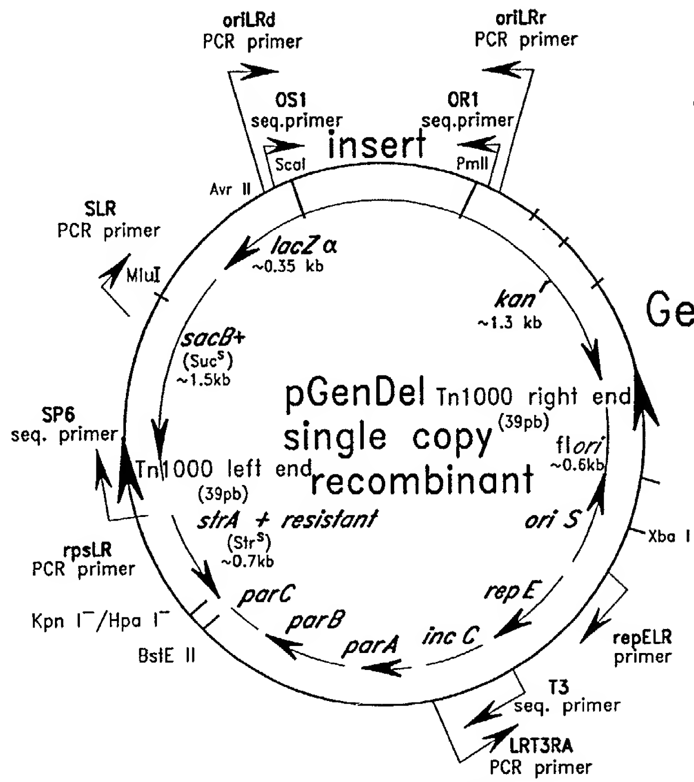


FIG. 4

FIG. 4A
FIG. 4B
FIG. 4C

Generation of nested deletions with Mung bean nuclease

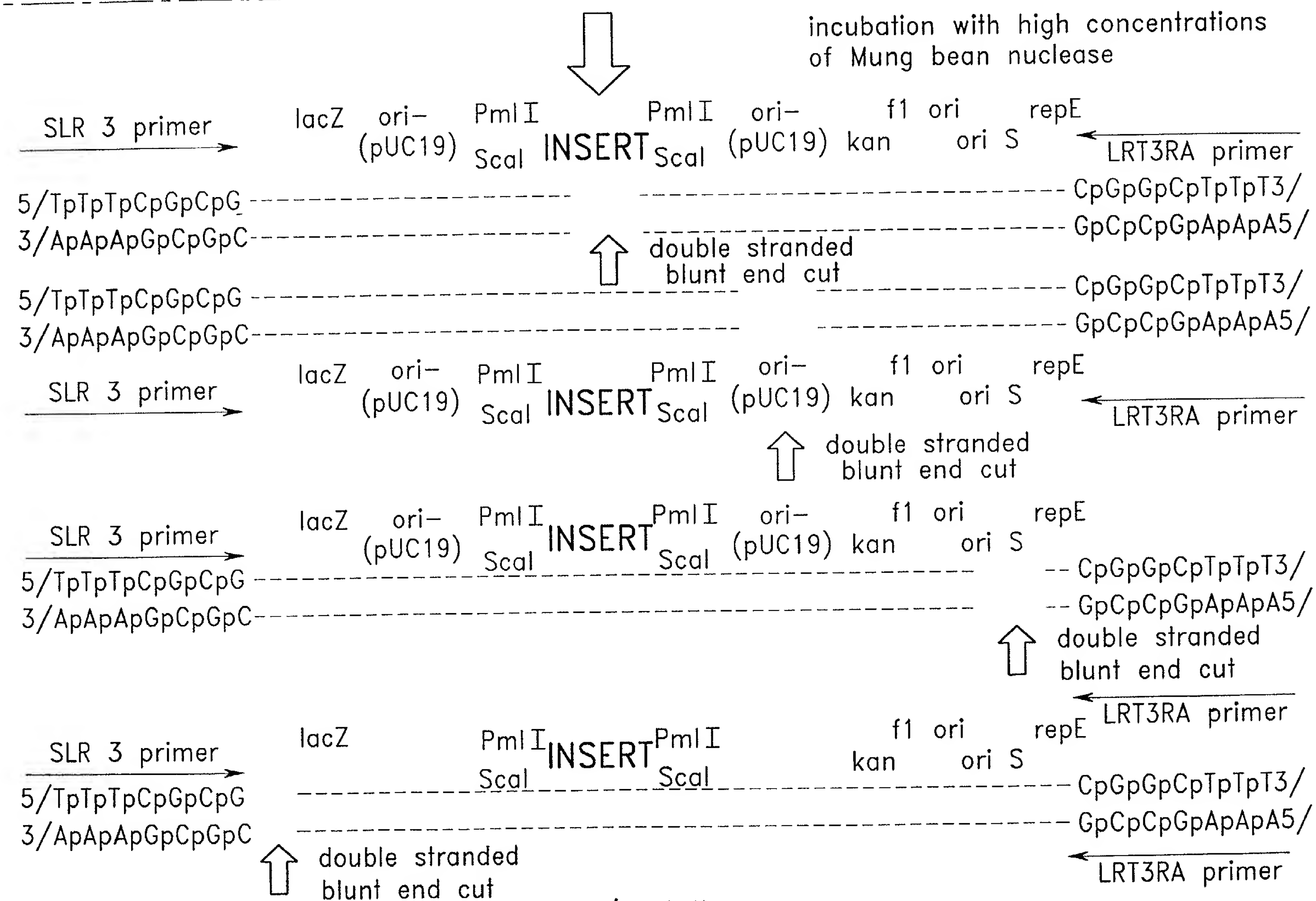
kanamycine resistant, streptomycine resistant if introduced into streptomycine resistant cells, sucrose sensitive, faint blue on IPTG/Xgal plates

generation of linear substrates by LR PCR with SLR3 and LRT3RA primers from cells

5/TpTpTpCpGpCpG ------------------------------------ CpGpGpCpTpTpT3/
3/ApApApGpCpGpC------------------------------------ GpCpCpGpApApA5/

SLR 3 primer → lacZ ori-(pUC19) PmlI ScaI INSERT PmlI ScaI ori-(pUC19) kan f1 ori ori S repE ← LRT3RA primer

FIG. 4A



incubation with high concentrations of Mung bean nuclease
SLR 3 primer
lacZ
ori-(pUC19)
PmlI
ScaI
INSERT
PmlI
ScaI
ori-(pUC19)
kan
f1 ori
ori S
repE
LRT3RA primer
5/TpTpTpCpGpCpG
3/ApApApGpCpGpC
CpGpGpCpTpTpT3/
GpCpCpGpApApA5/
double stranded blunt end cut

FIG.4B

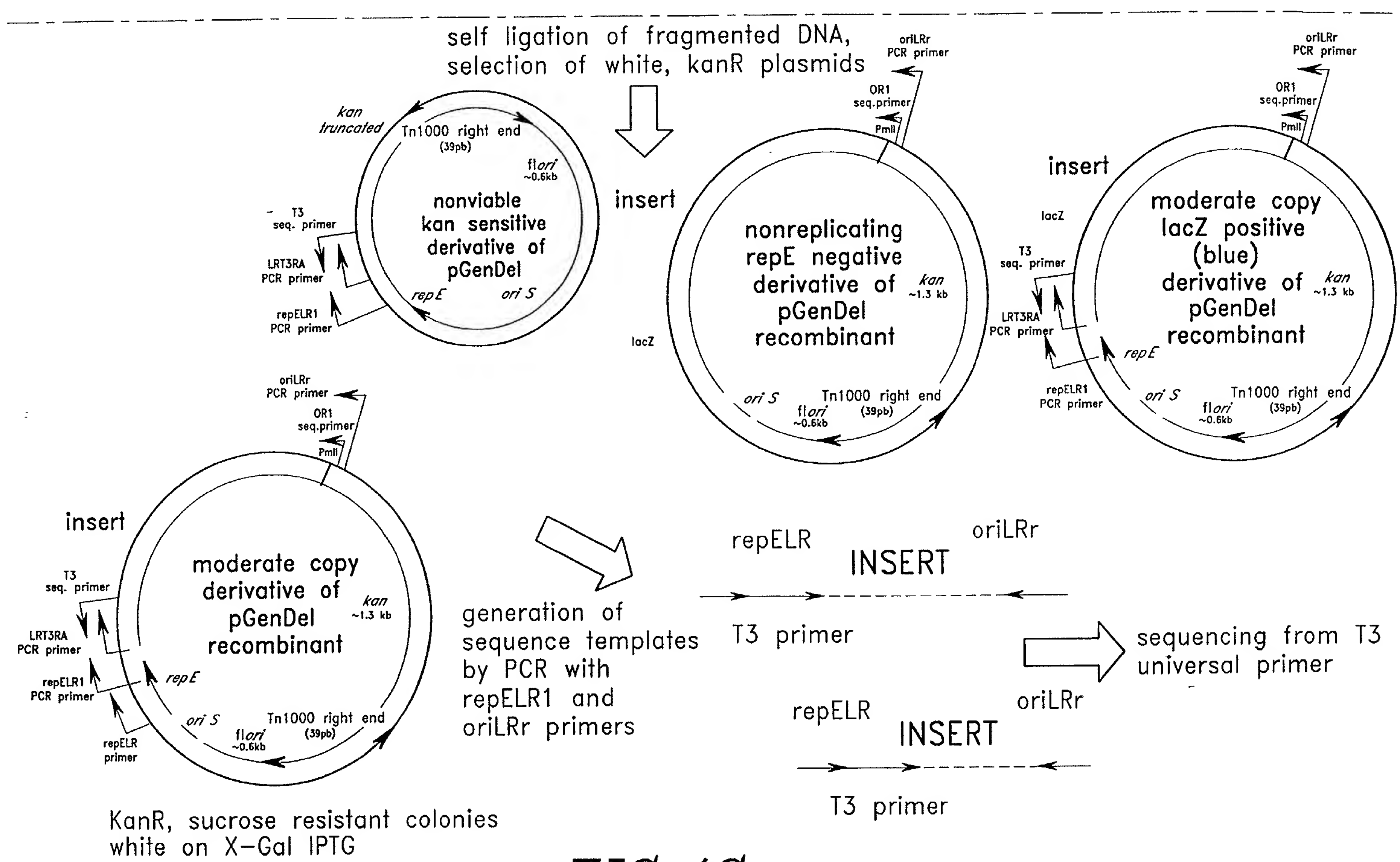
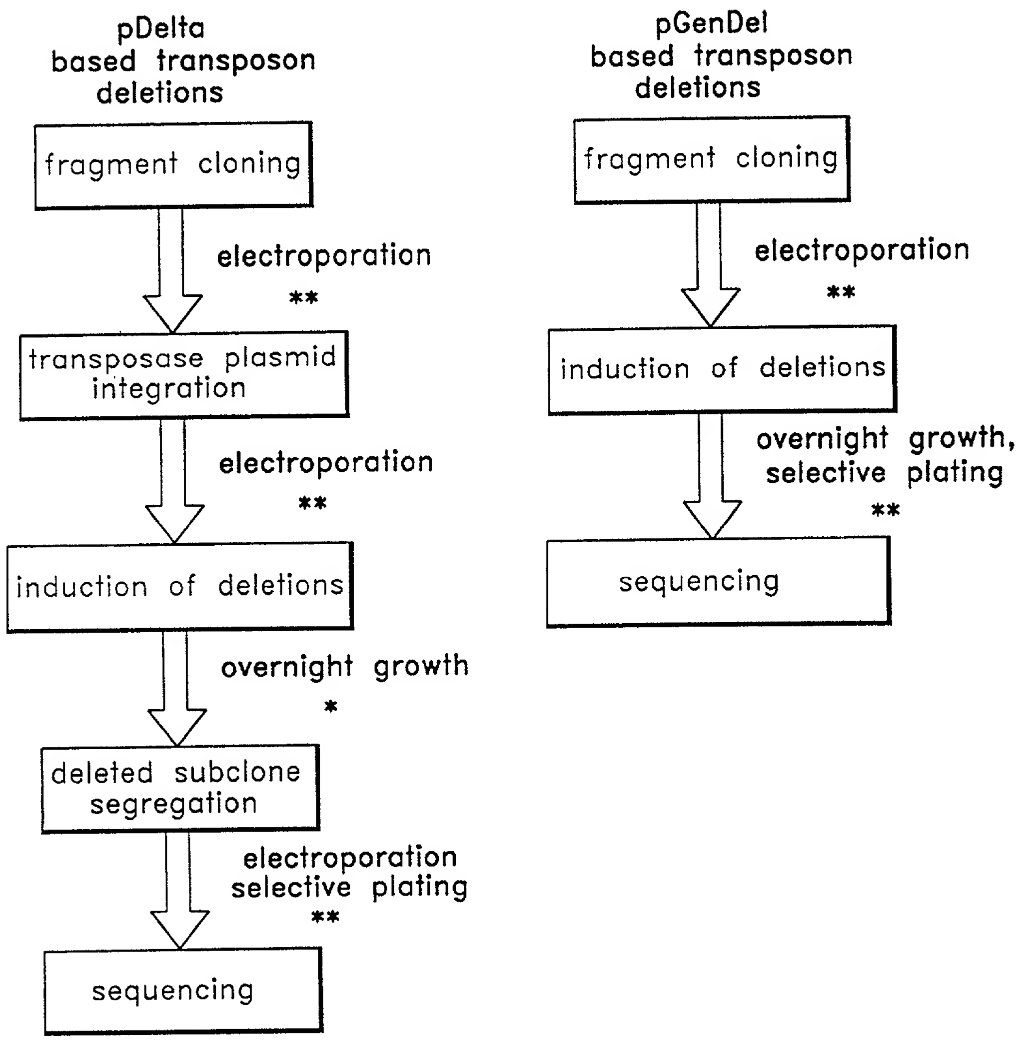

self ligation of fragmented DNA,
selection of white, kanR plasmids
kan truncated
Tn1000 right end (39pb)
flori ~0.6kb
nonviable kan sensitive derivative of pGenDel
repE
ori S
T3 seq. primer
LRT3RA PCR primer
repELR1 PCR primer
insert
lacZ
oriLRr PCR primer
OR1 seq.primer
PmlI
nonreplicating repE negative derivative of pGenDel recombinant
kan ~1.3 kb
moderate copy lacZ positive (blue) derivative of pGenDel recombinant
moderate copy derivative of pGenDel recombinant
repELR primer
KanR, sucrose resistant colonies
white on X-Gal IPTG
generation of sequence templates by PCR with repELR1 and oriLRr primers
repELR
INSERT
oriLRr
T3 primer
sequencing from T3 universal primer

FIG.4C

FIG. 5 | FIG. 5A | FIG. 5B

Comparison of different methods of nested deletion sequencing



*shown in *– easy stages
**– difficult stages
***–very difficult stages

FIG. 5A

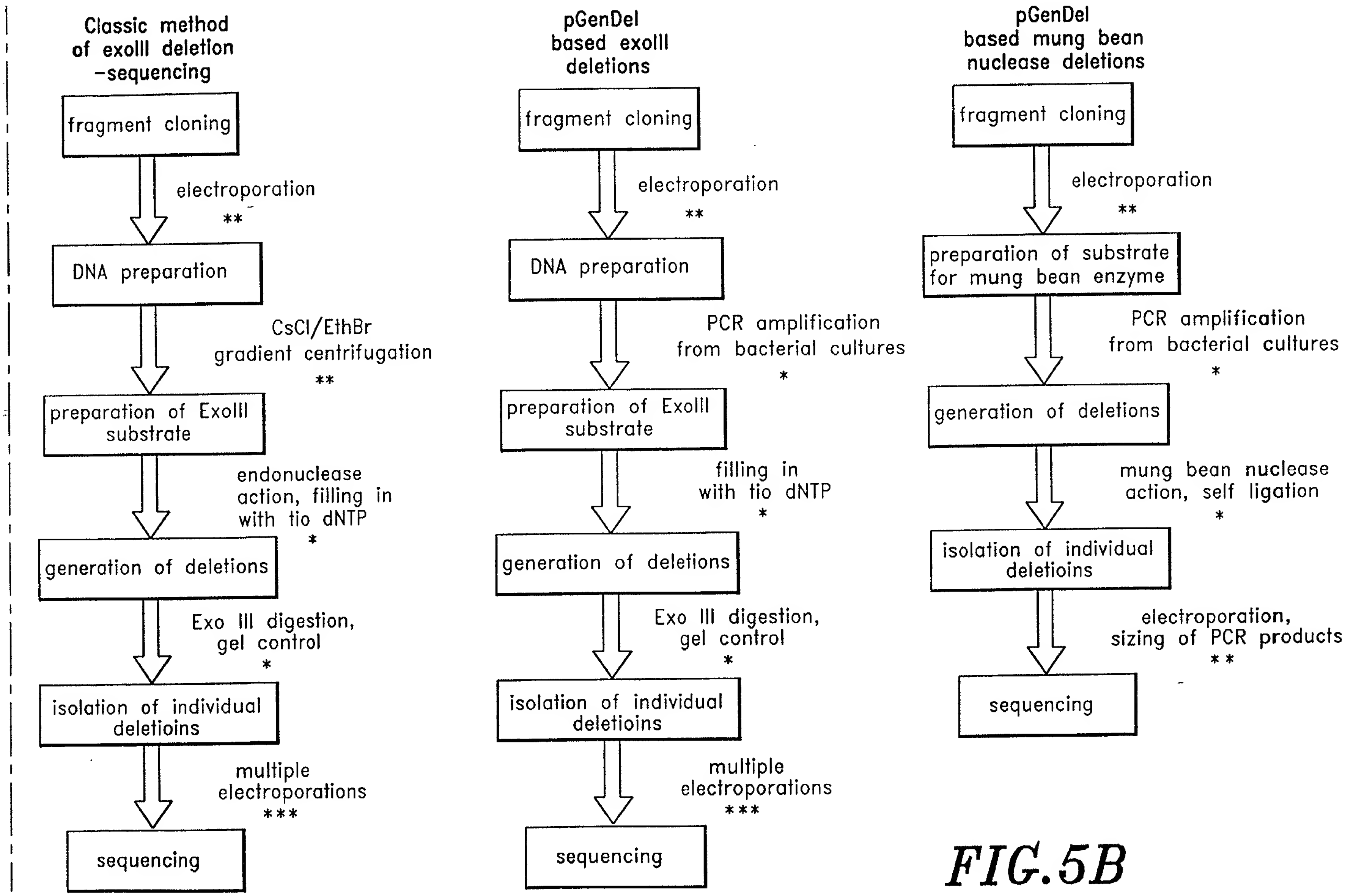

Classic method of exoIII deletion -sequencing
fragment cloning
electroporation **
DNA preparation
CsCl/EthBr gradient centrifugation **
preparation of ExoIII substrate
endonuclease action, filling in with tio dNTP *
generation of deletions
Exo III digestion, gel control *
isolation of individual deletioins
multiple electroporations ***
sequencing
pGenDel based exoIII deletions
fragment cloning
electroporation **
DNA preparation
PCR amplification from bacterial cultures *
preparation of ExoIII substrate
filling in with tio dNTP *
generation of deletions
Exo III digestion, gel control *
isolation of individual deletioins
multiple electroporations ***
sequencing
pGenDel based mung bean nuclease deletions
fragment cloning
electroporation **
preparation of substrate for mung bean enzyme
PCR amplification from bacterial cultures *
generation of deletions
mung bean nuclease action, self ligation *
isolation of individual deletioins
electroporation, sizing of PCR products **
sequencing

FIG.5B

THE SHOTGUN STRATEGY

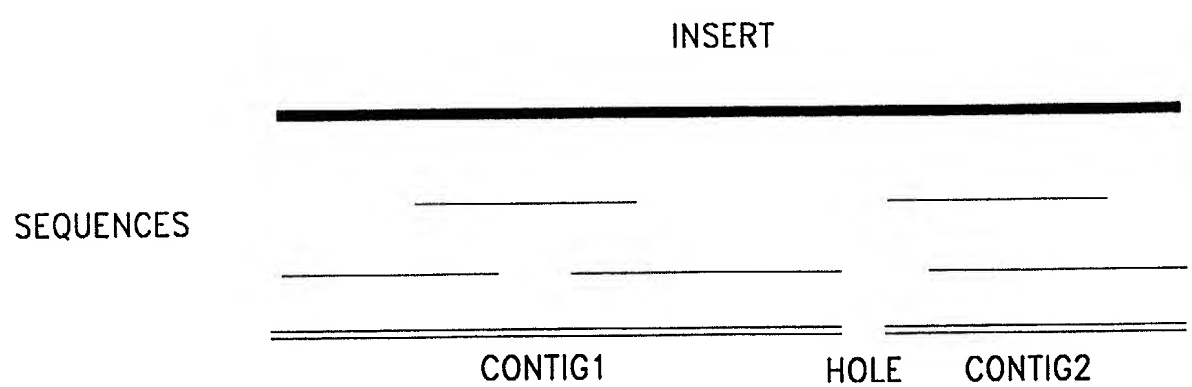


FIG.6

THE PAIRWISE STRATEGY

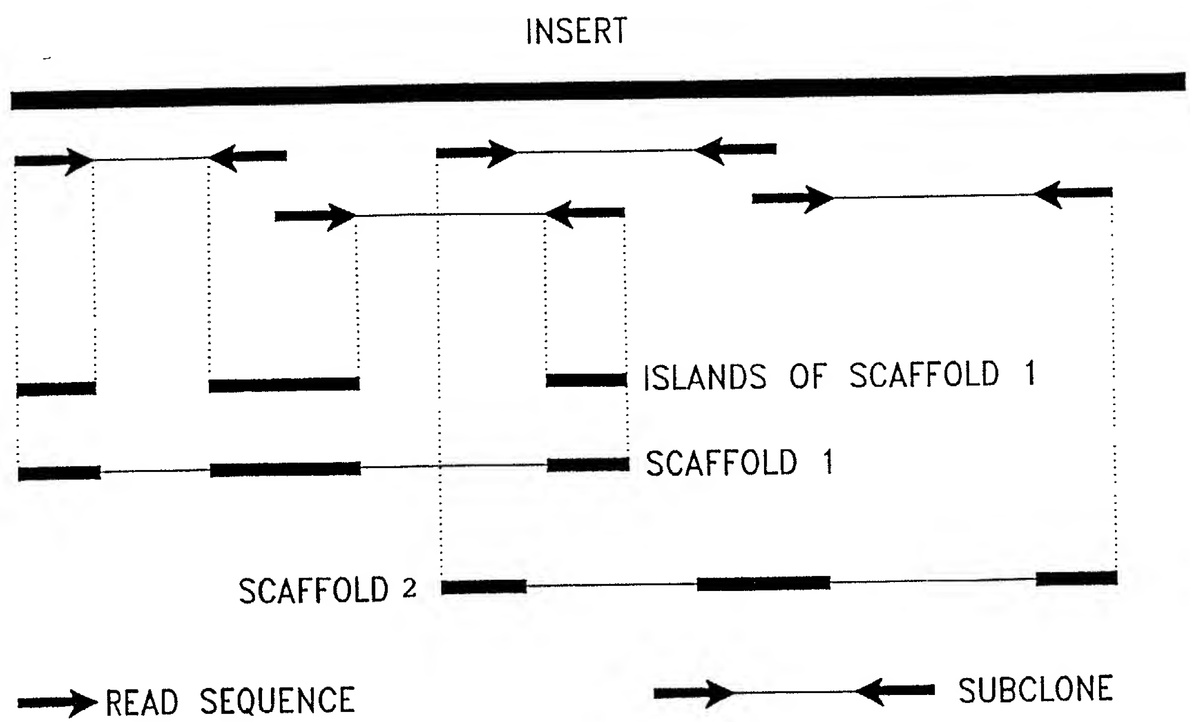


FIG.7

MULTIPLE NUCLEATION POINT

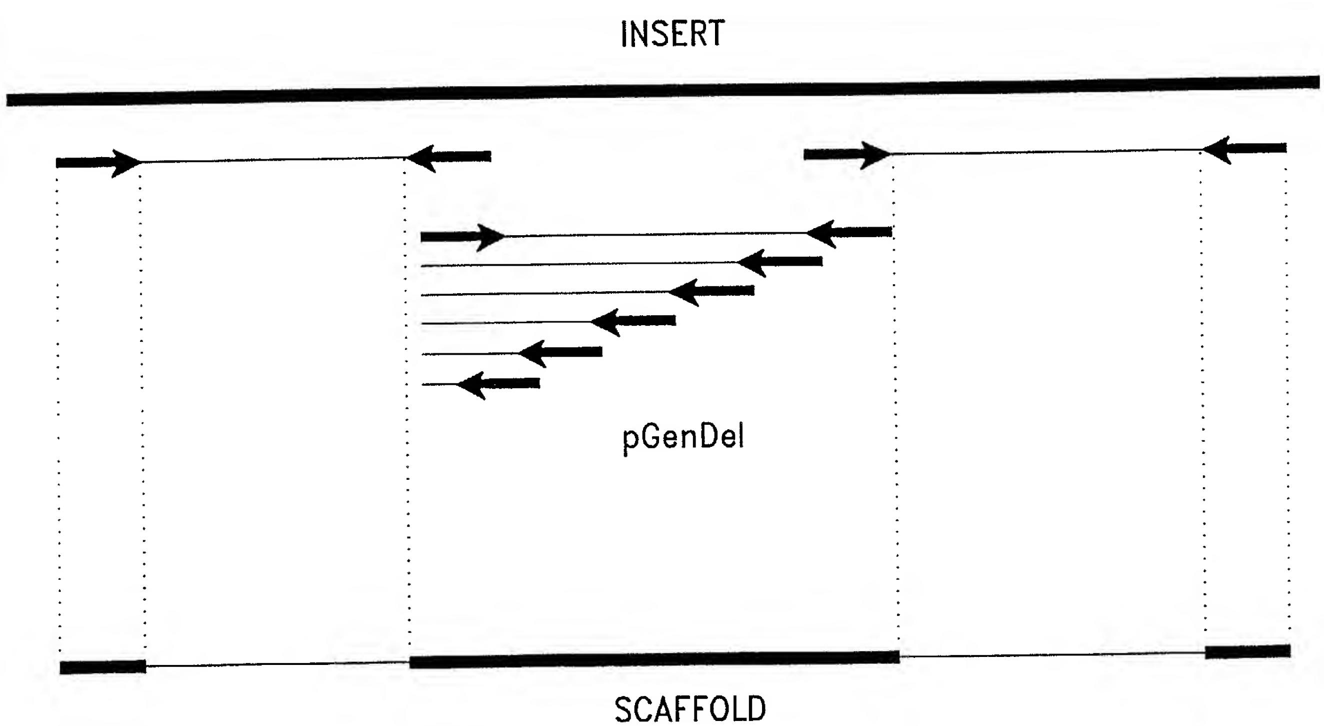


FIG.8

STRATEGIES FOR SEQUENCING OF LARGE DNA FRAGMENTS

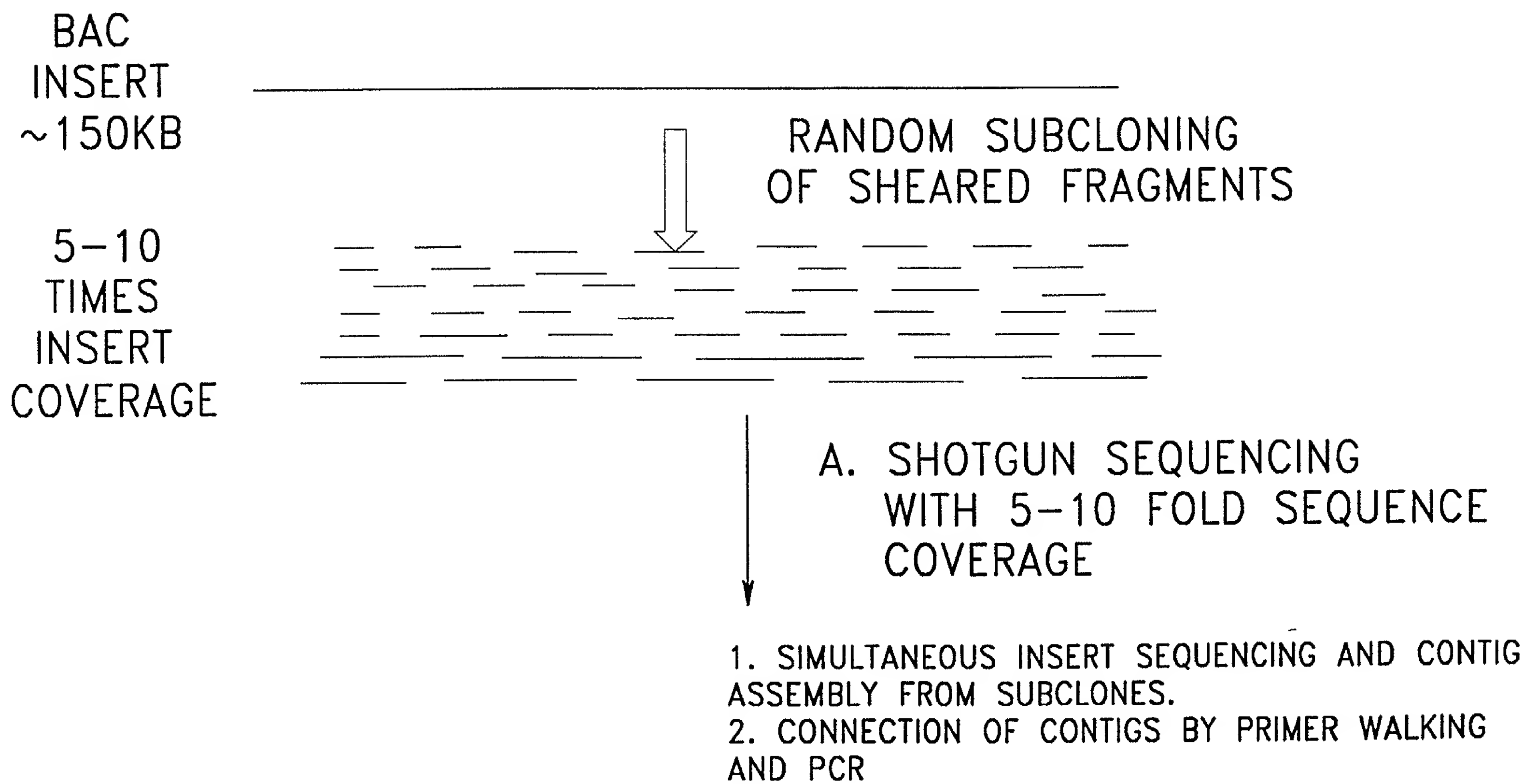


FIG. 9A

B. ORDERED SHOTGUN SEQUENCING–OSS

1. SIMULTANEOUS SEQUENCING OF BOTH ENDS OF LIMITED NUMBER OF SUBCLONES(1.5–2 FOLD SEQUENCE COVERAGE).
2. ASSEMBLY OF MINIMAL TILING PATH OF SUBCLONES BY PAIRWISE SEQUENCE OVERLAP.
3. PRIMER WALKING FOR EXTENSIVE SEQUENCING OF MINIMAL TILING PATH SUBCLONES

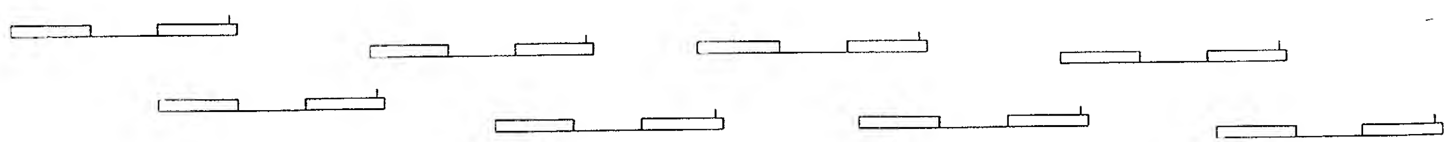


FIG.9B

C. MULTIPLE NUCLEATION POINT WALKING STRATEGY

1. SIMULTANEOUS COMPLETE SEQUENCING OF LIMITED NUMBER OF LARGE INSERT SIZE SUBCLONES WITH PAIRWISE END SEQUENCING FOR THE REST OF THEM RESULTS IN MINIMAL TILING PATH CONTAINING NUCLEATION POINTS OF HIGH QUALITY SEQUENCE.
2. TRANSPOSON MEDIATED SEQUENCING OF MINIMAL TILING PATH.

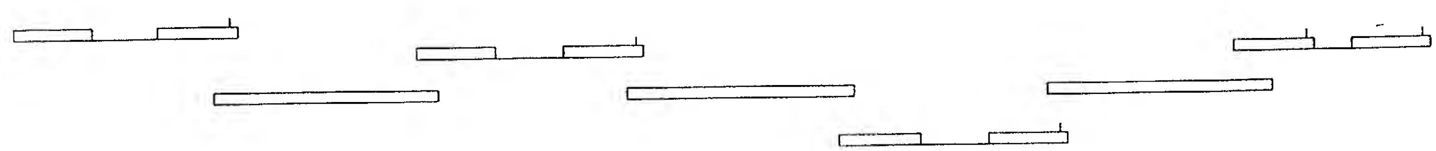


FIG.9C

	0	50	150	200	250	300	350	400	450	500	550	600	650	700	750	800
PAIRWISE ONLY	29023,2	45244,2	58801,3	68316,9	75206,4	79971,4	83504,1	85720,1	87923,1	88876,5	89630	90447,7	91191,6	91627,5	91925,6	92286,5
MULTIPLE NUCLEATION POINT	0	0	65553,8	83342,9	90466	93252,9	94234,1	94791,3	95127,1	95519,8	95770,1	96043,3	96178,7	96361,7	96443,5	96591,5

THE MAXIMUM SCAFFOLD LENGTH

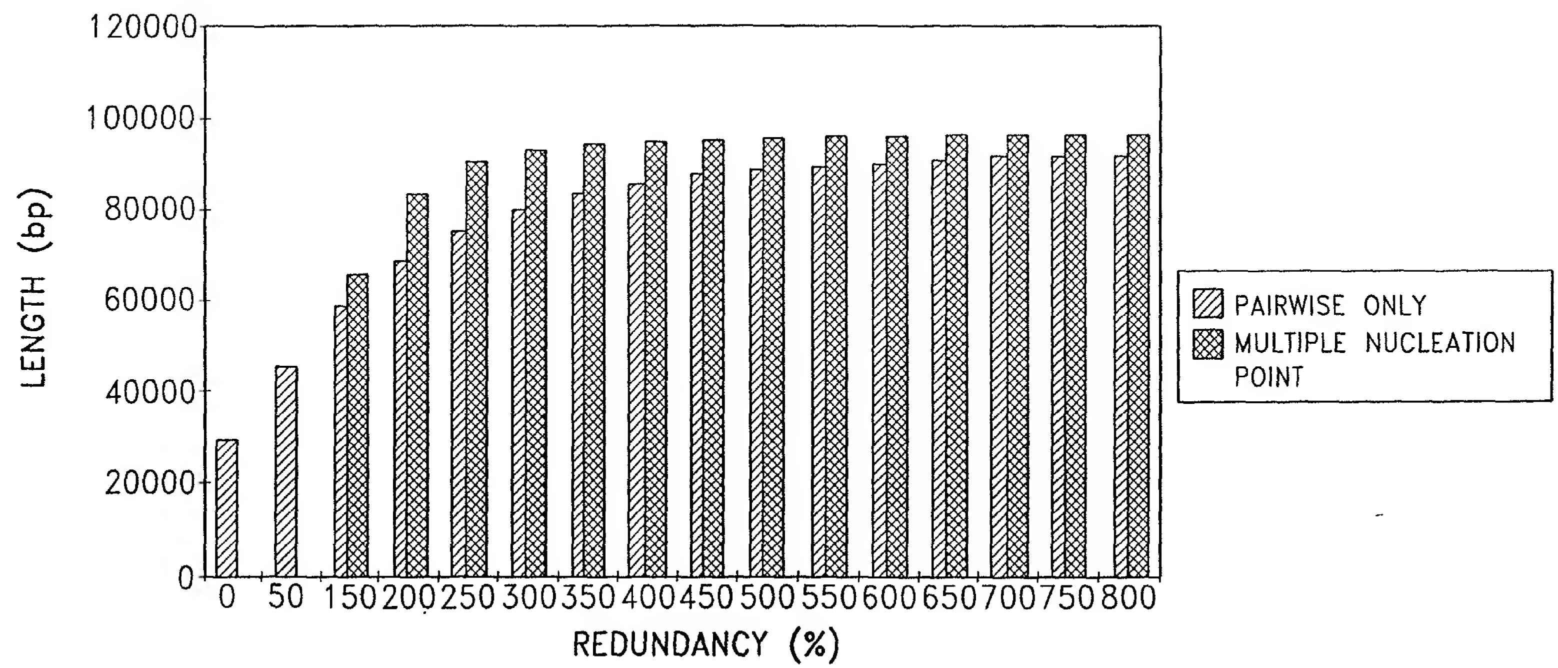


FIG.10

	50	100	150	200	250	300	350	400	450	500	550	600	650	700	750	800
PAIRWISE ONLY	8,908	16,455	17,729	15,476	12,483	9,701	7,522	6,11	4,974	4,208	3,662	3,24	2,894	2,655	2,467	2,274
MULTIPLE NUCLEATION POINT	0	0	2,5	2,338	2,185	2,046	1,931	1,808	1,676	1,577	1,484	1,43	1,374	1,332	1,302	1,264

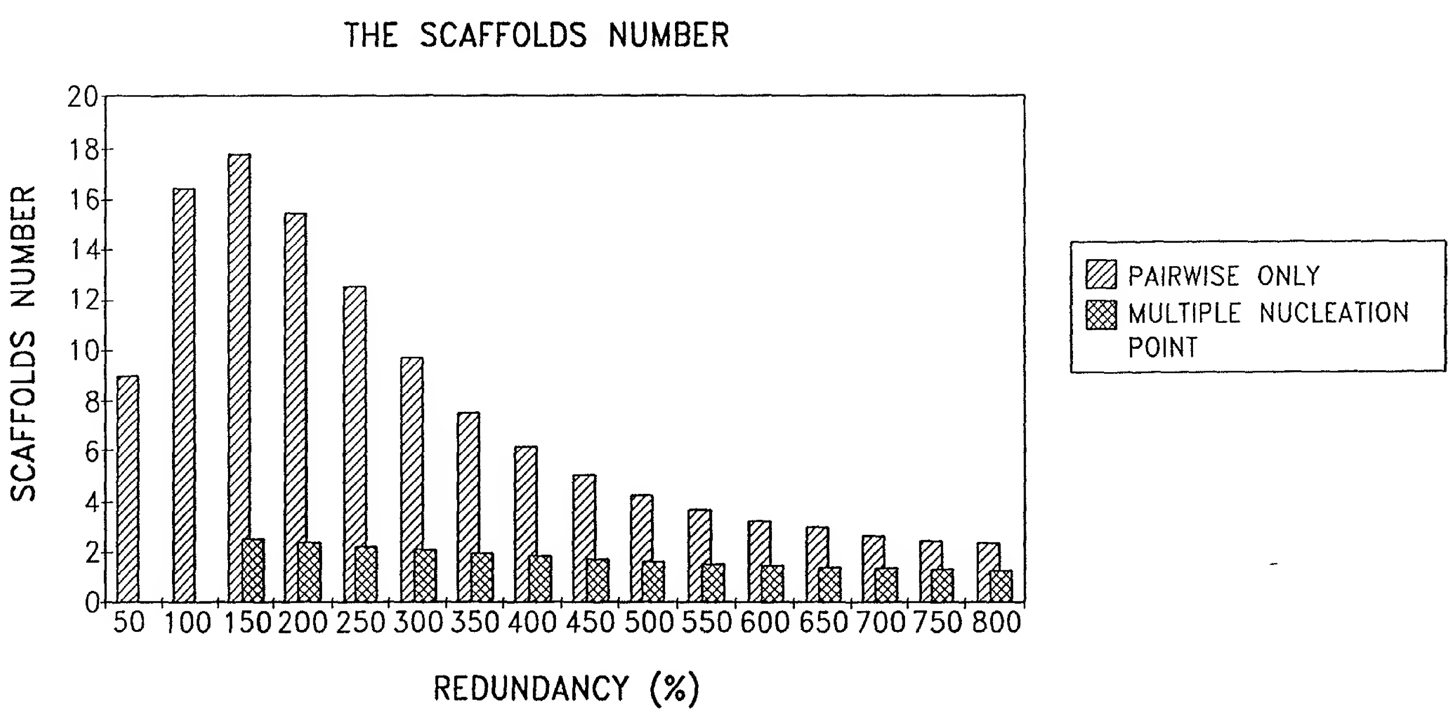


FIG. 11

	50	100	150	200	250	300	350	400	450	500	550	600	650	700	750	800
PAIRWISE ONLY	2,02868	2,64877	3,13585	3,67497	3,77673	3,72902	3,33939	3,00032	2,60141	2,31273	2,12644	1,92935	1,76033	1,64194	1,53327	1,41807
MULTIPLE NUCLEATION	0	0	1,20333	1,59241	1,65915	1,63765	1,53826	1,42167	1,30039	1,16708	1,01968	0,94504	0,83793	0,78088	0,74216	0,66806

STANDARD DEVIATION OF THE SCAFFOLDS NUMBER

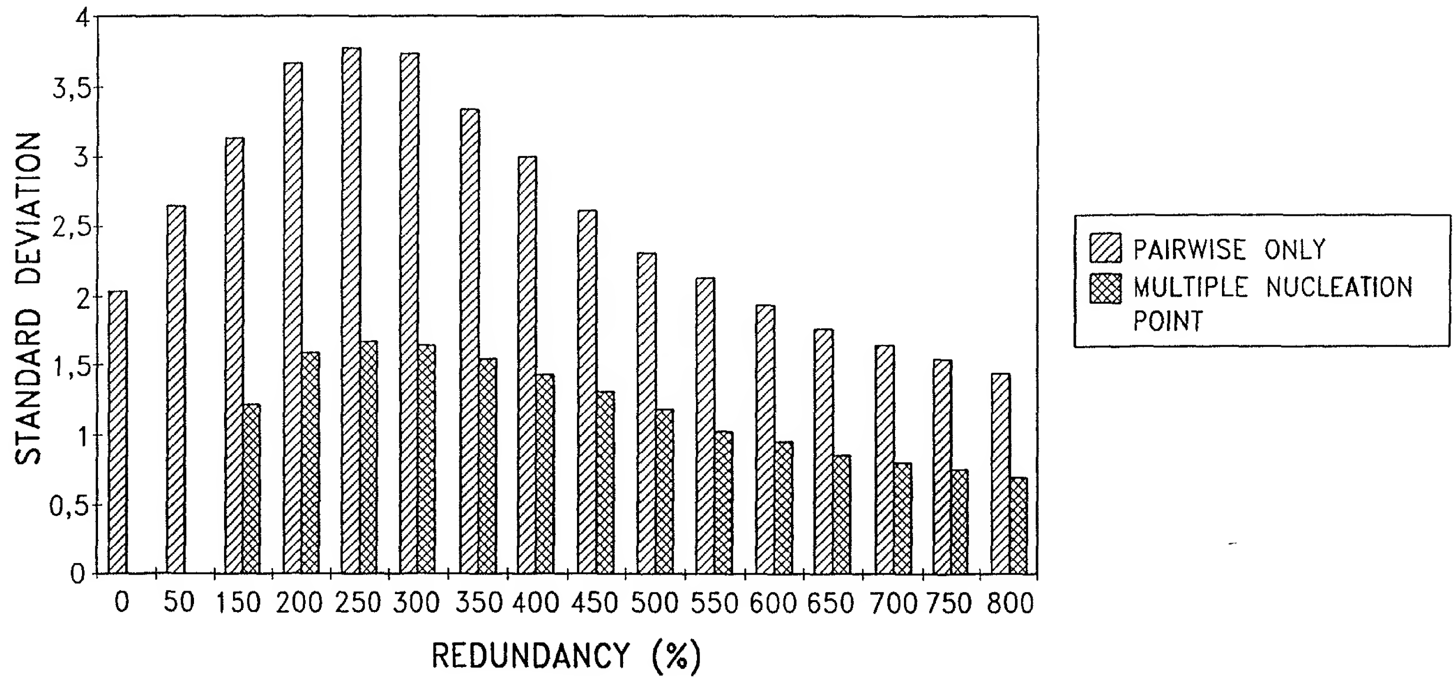
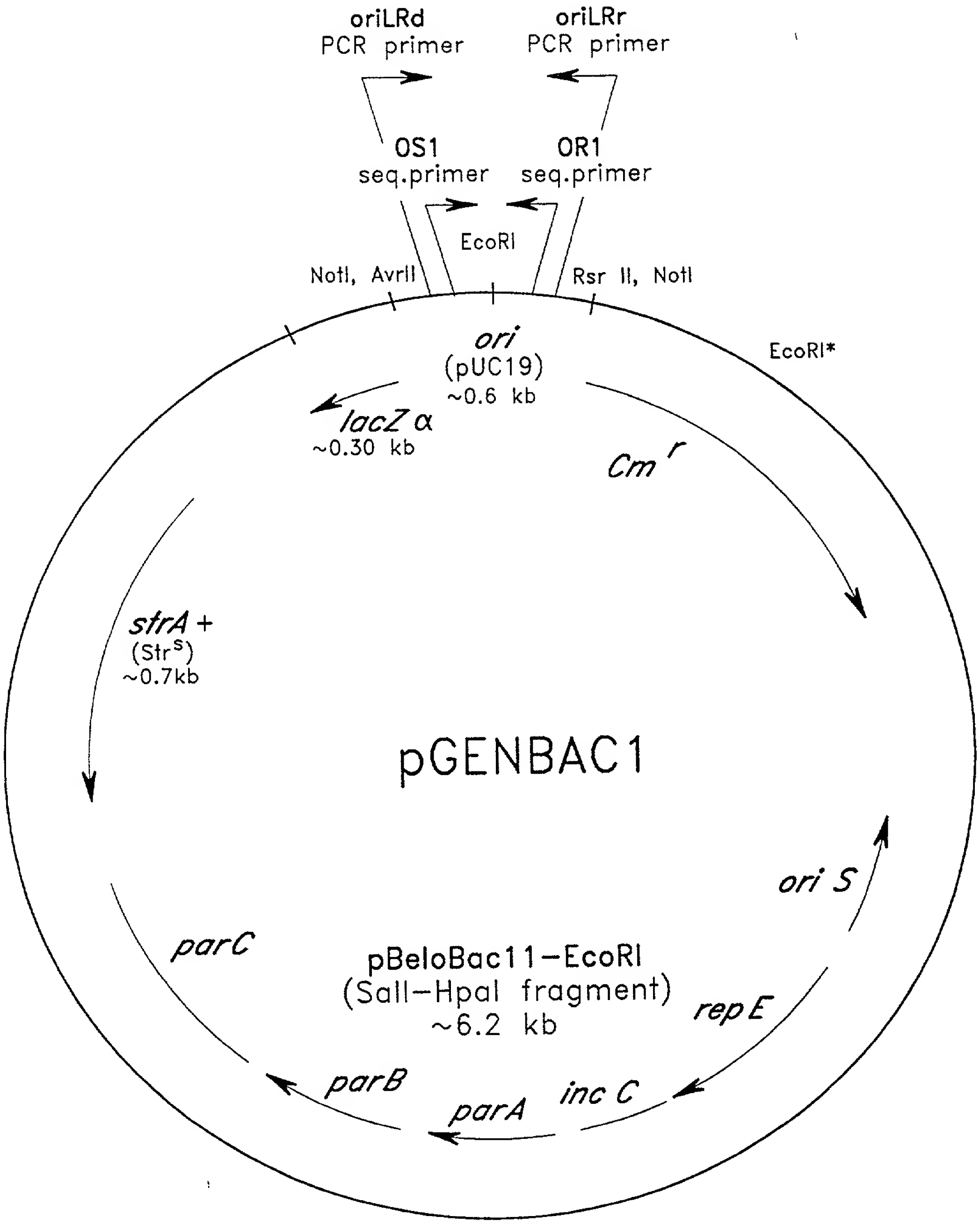


FIG. 12



oriLRd
PCR primer
oriLRr
PCR primer
OS1
seq.primer
OR1
seq.primer
EcoRI
NotI, AvrII
Rsr II, NotI
EcoRI*
ori
(pUC19)
~0.6 kb
lacZ α
~0.30 kb
Cm r
strA +
(Str S)
~0.7kb
pGENBAC1
ori S
parC
pBeloBac11-EcoRI
(SalI-HpaI fragment)
~6.2 kb
rep E
parB
parA
inc C

FIG. 13

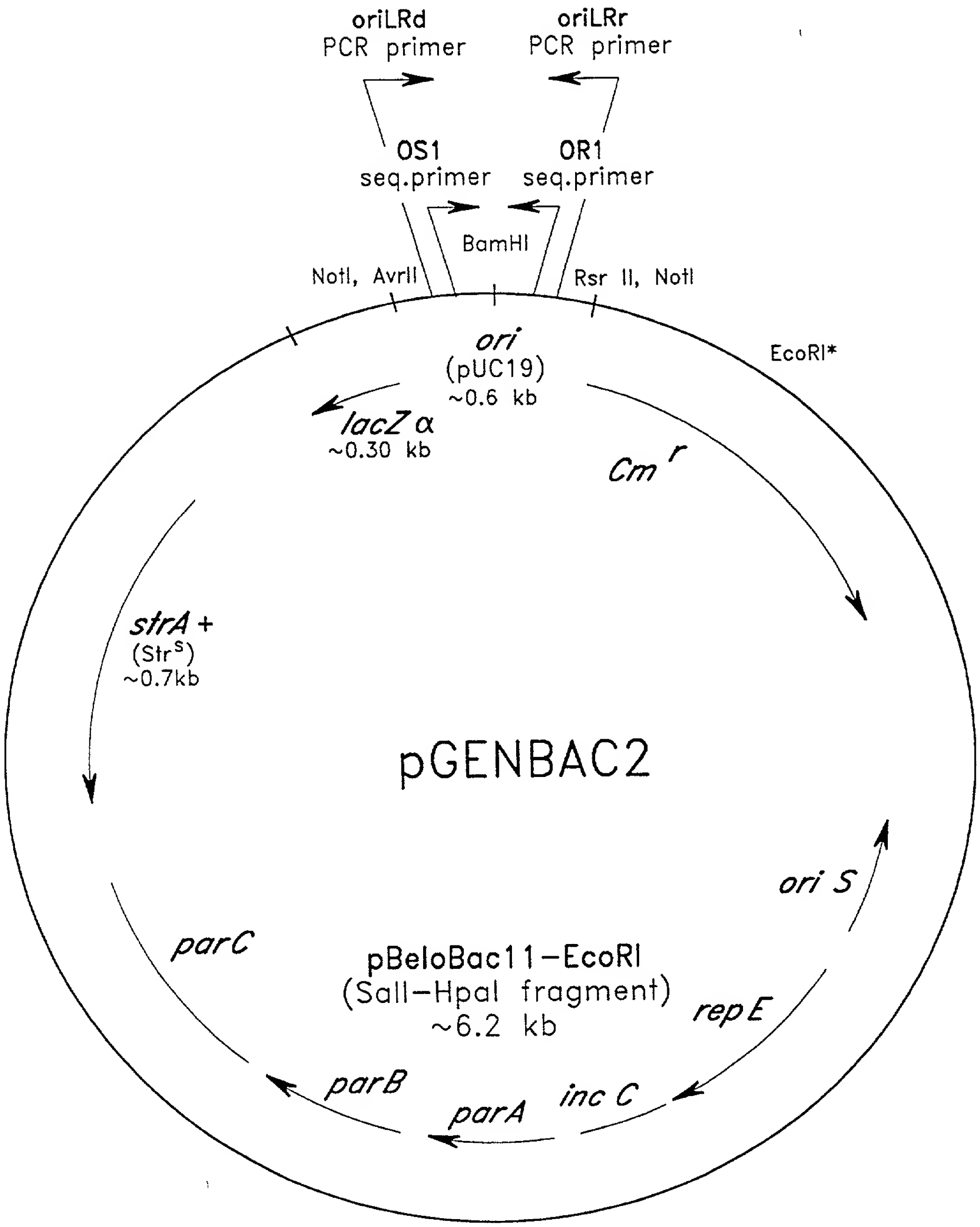

oriLRd
PCR primer
oriLRr
PCR primer
OS1
seq.primer
OR1
seq.primer
BamHI
NotI, AvrII
Rsr II, NotI
EcoRI*
ori
(pUC19)
~0.6 kb
lacZ α
~0.30 kb
Cm r
strA +
(Str s)
~0.7kb
pGENBAC2
ori S
parC
pBeloBac11-EcoRI
(SalI-HpaI fragment)
~6.2 kb
rep E
parB
parA
inc C

FIG. 14